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A novel organic-inorganic hybrid monolith for trypsin immobilization

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In proteomics, attention has focused on various immobilized enzyme reactors (IMERs) for the realization of high throughput digestion. In this report, a novel organic-inorganic hybrid monolith based IMER was prepared in a 100 μm i.d. capillary with 3-glycidoxypropyltrimethoxysilane (GLYMO) as the monomer and tetraethoxysilane (TEOS) as the crosslinker. Trypsin immobilization was achieved via the reaction between vicinal diol groups, which were obtained from hydrolysis of epoxy groups, and the amino groups of trypsin. Bovine serum albumin was digested thoroughly by this IMER in 47 s. After micro-reverse phase liquid chromatography-tandem mass spectrometry (μRPLC-MS/MS) analysis and database searching, beyond 35% sequence coverage was obtained, and the result was comparable to that of 12 h in solution digestion. The present IMER has potential for high throughput digestion.

immobilized enzyme reactor, organic-inorganic hybrid monolith, protein identification, HPLC-MS/MS

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Shot gun proteomics is an important analytical approach. High efficiency enzymatic digestion is crucial for this approach. Traditionally, this digestion is performed in various buffer solutions [1,2], and effective digestion requires a long digestion time (4–24 h). Real protein mixtures are very complex compared to individual proteins, which means they required even longer digestion time and higher enzyme to substrate ratios. As proteomics develops, automatic and high throughput analysis is becoming popular. Therefore, new digestion methods are required to replace solution digestion.

Enzyme immobilization [3] is a promising strategy for high throughput digestion. It allows the digestion time to be reduced to a few minutes or even seconds. For online digestion systems, manual handling is minimized because of direct connection between the immobilized enzyme reactors (IMERs) and other related subassemblies. In addition, the IMERs can be reused hundreds of times. To date, many IMERs [4–12] have been designed and applied successfully. Monolith based IMERs [8–12] prepared in capillaries have been particularly popular because of the monolithic relatively high binding capacity for enzymes, low backpressure, biological inertia, and mechanical stability.

In our previous work, an organic-inorganic hybrid IMER [13] based on amino groups for enzyme immobilization was developed, and showed high digestion activity. Here, another organic-inorganic hybrid IMER with classical epoxy groups was prepared to achieve more direct trypsin immobilization. The digestion performance demonstrated that this IMER had superior digestion activity and stability to our

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previous IMER.

1 Materials and methods

1.1 Materials and chemicals

The fused-silica capillary (100 μm i.d.×375 μm o.d.) was purchased from Sino Sumtech (Hebei, China). Tetraethoxysilane (95%, TEOS) and 3-glycidoxypropyltrimethoxysilane (>97%, GLYMO) were obtained from Acros organics (Geel, Belgium). Chitosan was supplied by Potuo Biomedical Corp. Polyethylene glycol 20000 (PEG 20000) was used as the porogen. A synthetic decapeptide, C-myc (97%, EQKLISEEDL), was ordered from Dalian Biotech Company Ltd. (Dalian, China). *L*-(Tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) treated trypsin (bovine pancreas), bovine serum albumin (BSA, bovine serum), cytochrome *c* (horse heart), apo-transferrin (bovine), sodium azide, iodoacetamide, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All organic solvents were HPLC grade. Water was purified by a Milli-Q system (Millipore, Molsheim, France).

1.2 Instrumentation

A precise syringe pump (Baoding Longer Pump Company, Baoding, China) was used to push samples through the IMERs, and a temperature controller (ZW-column oven, Dalian Elite Analytic Instruments Co., Ltd.) was utilized to maintain the temperature at 37°C. All conventional chromatographic experiments were performed in a high-performance liquid chromatograph (HPLC) system equipped with a UV detector (Jasco, Tokyo, Japan). An Allegra 64R centrifuge (Beckman coulter. Inc., Miami, USA) was used for sample purification. A μcapillary reverse phase liquid chromatography-electrospray ionization-tandem mass spectrometry (RPLC-ESI-MS/MS) system was constructed by combining nanoRPLC with a Finnigan LCQ XL IT mass spectrometer (Thermo-Fisher, San Jose, CA, USA).

1.3 IMER preparation

TEOS (672 μL) was mixed with 200 μL of methanol and 10 μL of 0.5 mol L^{-1} HCL until the solution was homogeneous. The solution was placed in a water bath at 60°C for 15 min. The solution was cooled to room temperature and H_2O (216 μL) was added. This solution reacted in a water bath at 60°C for 4 h. The TEOS sol was stored in the refrigerator at –20°C. Chitosan solution was added to improve the monolithic hydrophilicity. To make the chitosan solution 60 mg of chitosan was dissolved in 5940 μL of doubly distilled H_2O and 60 μL of pure acetic acid. This solution was vortex mixed for 5 min, and then sonicated for 10 min. The solution was centrifuged at 4000 r min^{-1} for 30 min, and the supernatant was collected and stored at –80°C (5 mL). The chitosan solution was poured into a 50 mL flask chilled in an ice bath to 0°C. GLYMO (470 μL) was added dropwise to the chitosan solution with slowly electromagnetic agitation. The solution was reacted at 65°C for 18 h with electromagnetic agitation. The GLYMO sol (90 μL) was mixed with 8 mg of PEG20000 and vortexed for 20 s, and sonicated for 10 min to obtain a homogeneous solution. This was followed by addition of 100 μL of the TEOS sol, vortex mixing for 20 s, and sonication for 10 min. The final solution was injected into a 100 μm i.d. capillary. After both ends of the capillary were sealed by silicon rubbers, it was placed at 40°C for 18 h. The capillary was subsequently rinsed with ethanol and water for 30 min each to elute the pore-forming solvent. Trypsin solution (2 mg mL^{-1}) in phosphate buffered saline (PBS) (100 mmol L^{-1}, pH 10.0) containing 0.05 mol L^{-1} benzamide was pumped into the capillary at 4°C for 4 h. The microreactor was stored at 4°C. For the IMER, the immobilized amount of trypsin was calculated by measuring the difference between the concentrations of enzyme in the supernatant before and after the immobilization by Bradford assay.

1.4 Protein digestion

BSA was dissolved in 50 mmol L^{-1} NH_4Ac (pH 8.0, 100 μL) containing 8 mol L^{-1} urea and then reduced in 10 mmol L^{-1} dithiothreitol for 1 h at 56°C. The solution was cooled to room temperature, and the proteins' cysteines were alkylated in the dark in 20 mmol L^{-1} iodoacetic acid for 30 min at 37°C. This was followed by the dilution with 50 mmol L^{-1} NH_4Ac (pH 8.0, 900 μL) to reduce the urea concentration to <1 mol L^{-1}. This solution was stored at –80°C. The in-solution digestion was performed by adding trypsin to the pretreated protein sample with a substrate-to-enzyme ratio of 50:1 (w/w) at 37°C overnight. Because of the large number of sequence-specific cleavage sites in BSA, we prolonged the incubation time to 12 h. Finally, 2 μL of formic acid was added to the solution to terminate the reaction.

1.5 HPLC and MS analysis

All experiments for quantitative analysis of C-myc were performed on an HPLC system equipped with a UV detector. The HPLC column (4.6 mm i.d.×150 mm, 5 μm, pore size 80 Å) was packed with octadecyl silane ODS-2. The mobile phase was (A) water with 0.1% trifluoroacetic acid (TFA) and (B) 95% acetonitrile (ACN) with 0.1% TFA at a flow rate of 1.0 mL min^{-1}. The mobile phase gradient from 0–30 min was 18%–22% B. The injection volume was 5 μL and the detection wavelength was 214 nm. Each measurement was repeated twice for precision. A μHPLC-ESI-MS/MS system for protein identification was constructed.

For standard proteins the HPLC conditions were as follows: injection volume, 2 μL; separation column, 300 μm i.d.×15 cm, 5 μm, pore size 300 Å; column packing material, ODS; mobile phase, (i) 2% ACN containing 0.1% formic acid, (ii) 98% ACN containing 0.1% formic acid; mobile phase flow rate, 5 μL min^{-1}; mobile phase gradient, 0 min 2% B, 10 min 2% B, 15 min 10% B, 55 min 40% B, 65 min 80% B, 70 min 80% B. The MS conditions were as follows: voltage, 3 kV; capillary temperature, 150°C; collision energy, 35%. After the peptides eluted from the microcapillary column, they were introduced directly into the ESI-MS.

1.6 Database searching

Total ion chromatograms and mass spectra were recorded on a PC equipped with Xcalibur software version 1.4 for LCQ^{DUO}. Mass calibration and tuning were performed in positive ion mode. One micro scan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. The MS was set as one full MS scan followed by three MS/MS scans on the three most intense ions for LCQ. The dynamic exclusion function was set as follows: repeat count 2, repeat duration 30 s, and exclusion duration 180 s. The tandem mass spectra analysis and protein database searching were operated with Bioworks 3.1 software for LCQ^{DUO}. Protein identification was performed using BioWorks 3.1 software for LCQ. We searched myoglobin and other standard proteins from the equine and bovine 2 databases. Cysteine residues were searched as static modification of 57.0215 Da. The peptides were filtered utilizing the standard: Xcorr was higher than 1.9 for singly charged peptides, 2.2 for doubly charged peptides, and 3.75 for triply charged peptides.

2 Results and discussion

2.1 Preparation and characterization of organic-inorganic monolith

Sol-gel chemistry is frequently used to prepare IMERs. In our earlier study, an organic-inorganic hybrid matrix was synthesized using TEOS and 3-aminopropyltriethoxysilane. This IMER was applied for trypsin immobilization, and was very effective at digesting proteins. In this report, we prepared an organic-inorganic hybrid IMER using TEOS and GLYMO as precursors. PEG20000 was selected as the porogen after optimization. Moreover, chitosan was added to improve the hydrophilicity. The preparation procedures are outlined in Figure 1. Because of the strong polarity of the

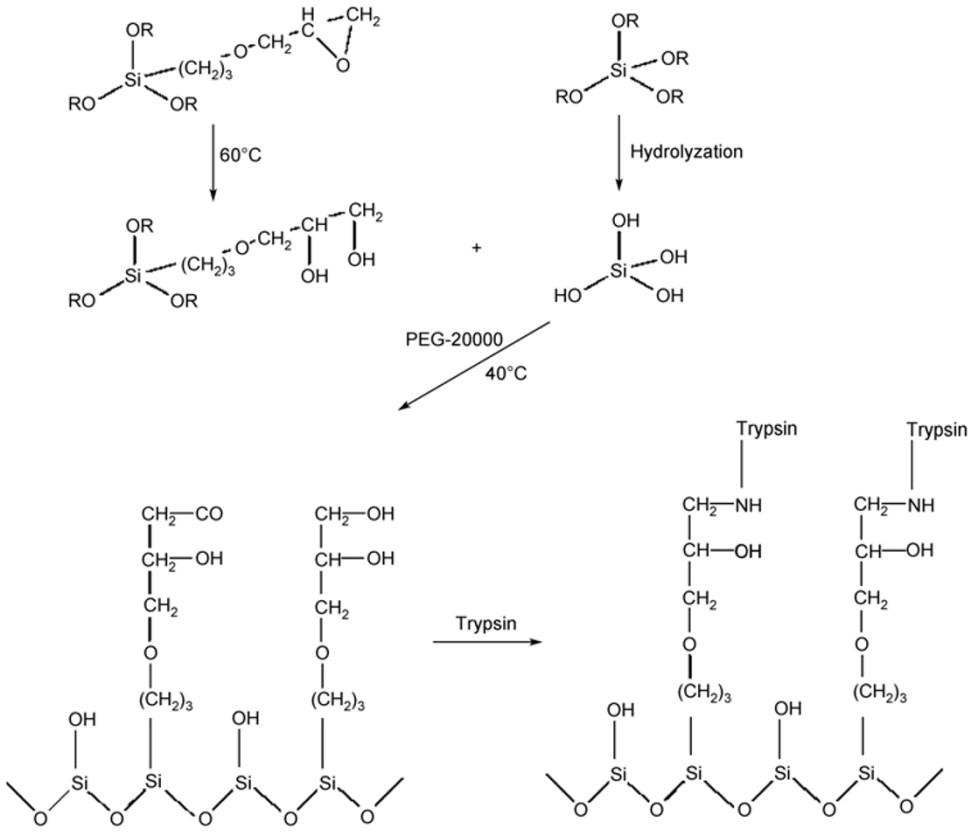


Figure 1 Schematic diagram for IMER preparation.

GLYMO molecules, a transparent gel was formed when GLYMO and TEOS were mixed directly for the sol-gel preparation. Consequently, the prepared capillary column is not very permeable. Therefore, the GLYMO and TEOS sols were hydrolyzed first, and then mixed together in an optimized proportion. Table 1 presents the optimization process in the last preparation phase. With 120 μL of GLYMO, sol phase separation did not occur, which resulted in single-phase solid gel that could not be tightly attached to the inner wall of the capillary. To address this problem, the volume of TEOS sol was increased from 90 to 110 μL. A satisfactory result was obtained with 100 μL of TEOS, but with more or less induced poor mechanical stability (column B) or unacceptable permeability (column D). Therefore, column C was synthesized, followed by trypsin immobilization. For accurate calculation of the immobilized enzyme amount, four C columns with identical lengths were prepared, and a series of standard protein solutions (pH 8.3) with different concentrations were used for calibration. The results demonstrated that on average 0.8 μg of trypsin was immobilized over 1 mm^3 of the sol-gel support.

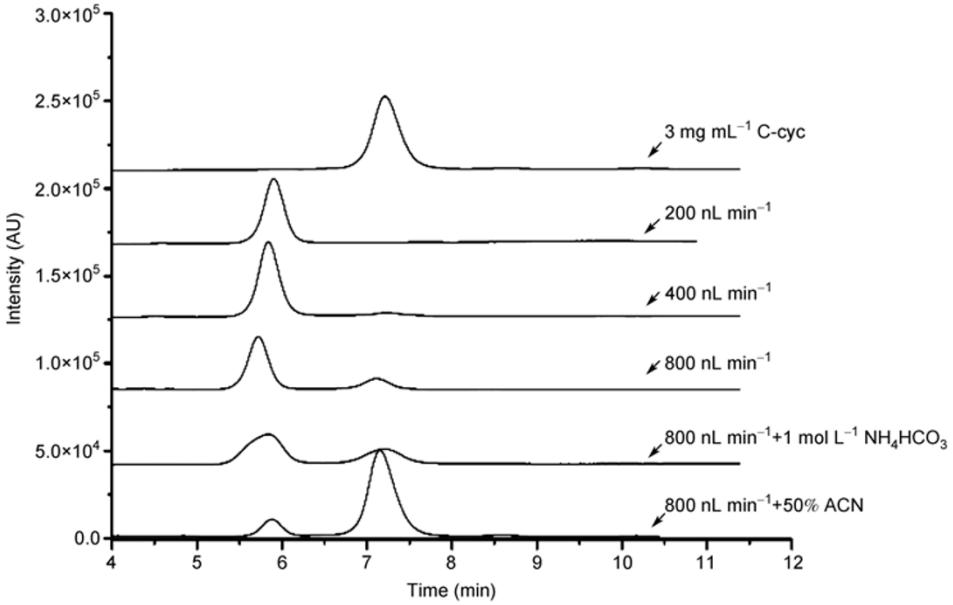
Table 1 Proportion optimization for monolithic matrix preparation

Monolith	GLYMO gel (μL)	TEOS gel (μL)	PEG20000 (mg)	Backpressure (MPa)	Mechanical intensity
A	120	70	8	<1	Poor
B	100	90	8	<1	Poor
C	90	100	8	1–5	Proper
D	80	110	8	>20	Hard

2.2 Digestion activity

The synthetic decapeptide C-myc, which can be efficiently cleaved by trypsin, was employed as the digestion substrate to test the digestion activity of the IMER. The digestion conditions included gradually increased flow rates and high concentrations of ACN and NH_4Ac. The relevant chromatograms are presented in Figure 2. Until a flow rate of 800 nL min^{-1} (1.7 mm s^{-1}), a peak for undigested C-myc appeared, which revealed digestion activity by trypsin. Afterward, 50% ACN was added to the substrate solution, and it was digested by the same capillary IMER at 800 nL min^{-1}. Unsurprisingly, large numbers of C-myc could not be digested in these harsh conditions. High concentrations of ACN can reduce the activity of enzymes, and even partly denature enzymes. Proteolytic digestion was then conducted in the presence of 1 mol L^{-1} NH_4Ac mixed with 3 mg mL^{-1} C-myc. The digestion result showed that the digestion activity of trypsin was recovered. The addition of 1 mol L^{-1} NH_4Ac did not have such serious impact on trypsin activity. Trypsin digestion still occurred in some degree in the presence of salts. Therefore, the sol-gel column is a robust matrix in terms of trypsin immobilization.

BSA (MW, 66 kD) was chosen as a model protein for further investigation of the digestion activity of trypsin immobilized into the organic-inorganic hybrid matrix. The flow rate through IMER or residence time of substrate to enzyme is a crucial factor that affects the digestion result. For the prepared IMER, the flow rates ranged from 100 to 500 nL min^{-1}. The corresponding residence times were from 0.8 to 3.9 min. The effluents from the microreactor were collected and analyzed by ESI-MS/MS. Figure 3 displays the detailed identification information of BSA tryptic digests using IMER (500 nL min^{-1}). As can be seen, the samples were clearly identified at flow rates from 100 to 500 nL min^{-1}. When the flow rate increased to 300 nL min^{-1} there was a transition in the relationship between the flow rate and sequence coverage or unique peptide number. The fac-



Figue 2 HPLC spectra of C-myc digestion. IMER, 5 cm, 100 μm i.d.

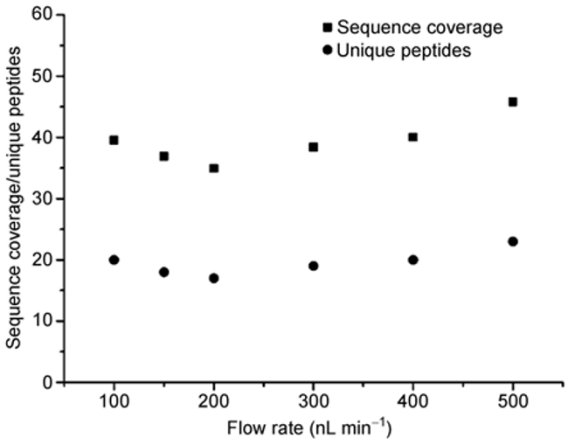


Figure 3 Influence of digestion flow rates on the digestion of BSA by IMER. Database searching was performed using *bovine2.fasta.* The sample injected was 2 μL of 1.47 nmol mL^{-1} BSA in 50 mmol L^{-1} NH_4Ac-NH_4OH buffer (pH 8.0). The IMER was 5 cm×100 μm i.d. The incubation time was 47 s in solution for 16 h. The trypsin to protein ratio was 1:50. Separation conditions are detailed in Materials and methods section.

tors inducing this transition probably include a change in residence time and mass diffusion resistance. At low flow rates, the residence time is the most important factor affecting digestion, and at high flow rates, the mass diffusion resistance predominates.

To investigate the repeatability of digestion with the IMER, three consecutive digestions were performed with a flow rate of 300 nL min^{-1}. All digestions used the same μRPLC-MS/MS system for separation. The relative standard deviation (RSD) of sequence coverage was calculated. The RSD was 3.9%, which is satisfactory for such a big protein. This result indicated that the stability of the digestion was relatively good.

2.3 *Escherichia coli* digestion

Freshly extracted *E. coli* cells (8 μg) [14] in a protein mixture were digested using the organic-inorganic hybrid IMER (10 cm length). It is well known that *E. coli* is a convincing model for investigation of IMER. The digestion products were separated and analyzed by ESI-MS/MS using strict multiple database searching thresholds. The corresponding RPLC chromatogram is presented in Figure 4. The 121 unique peptides and resultant 84 proteins were identified by database searching, with MW ranging from 4 to 180 kD and pI ranging from 4.5 to 10.5. With an increased sample injection amount, more proteins are expected to be identified. This result means that this IMER has a huge potential to achieve high throughput proteome digestion.

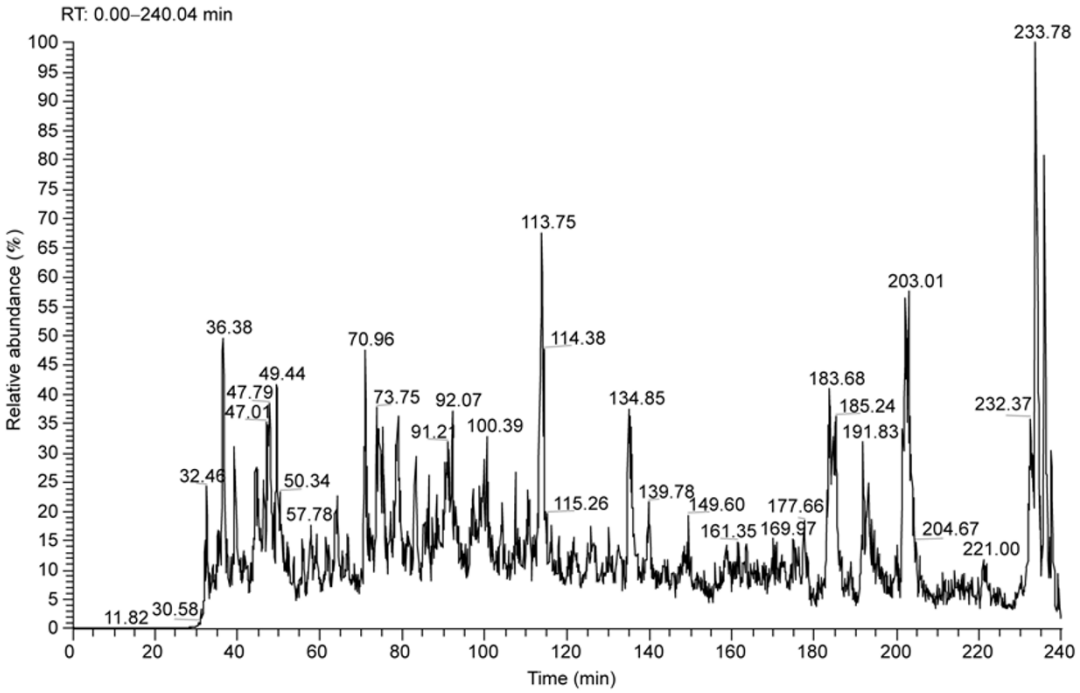


Figure 4 Base peak chromatogram of the *E. coli* extract. Digestion conditions: column, 10 cm×100 μm i.d.; mobile phase flow rate, 300 nL min^{-1}; temperature, 25°C. The *E. coli* protein concentration was 0.4 mg mL^{-1}, the injection volume was 20 μL, and the flow rate was 5 μL min^{-1}. This was followed by a 60 min elution with 0% B. The separation was performed using the following gradient conditions: 0%–10% B in 10 min, 10%–40% B in 200 min, and 40%–80% B in 10 min. The final wash of the C18 column was performed with 80% B for 20 min. The MS condition voltage was 2.0 kV. The LCQ-IT MS was operated in positive ion mode, and the capillary temperature was 150°C. Database searching was performed using *Final_e.coli.fasta*. RT, retention time.

3 Conclusion

An organic-inorganic hybrid monolith based IMER was prepared in capillary with a reliable mechanical stability and chromatography permeability. The trypsin activity was maintained. It digested *E. coli* proteins in 1 min. This IMER is promising for the development of an automated online digestion device in proteomics.

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1 Aaron A K, Michael J M. Effects of modified digestion schemes on the identification of proteins from complex mixtures. J Proteome Res, 2006, 5: 695–700
2 Hervey, Strader M B, Hurst G B. Comparison of digestion protocols for microgram quantities of enriched protein samples. J Proteome Res, 2007, 6: 3054–3061
3 Byrne R E, Scanu A M. Soluble and immobilized trypsin as structural probes of human plasma high-density lipoproteins: Enzyme properties and kinetics of proteolysis. Biochemistry, 1983, 22: 2894–2903
4 Li Y, Yan B, Deng C H, *et al.* Efficient on-chip proteolysis system based on functionalized magnetic silica microspheres. Proteomics, 2007, 7: 2330–2339
5 Li Y, Xu X Q, Deng C H, *et al.* Immobilization of trypsin on superparamagnetic nanoparticles for rapid and effective proteolysis. J Proteome Res, 2007, 6: 3849–3855
6 Li Y, Yan B, Deng C H, *et al.* On-plate digestion of proteins using novel trypsin-immobilized magnetic nanospheres for MALDI-TOF-MS analysis. Proteomics, 2007, 7: 3661–3671
7 Cooper G W, Chen J Z, Li Y, *et al.* Membrane-based nanoscale proteolytic reactor enabling protein digestion, peptide separation, and protein identification using mass spectrometry. Anal Chem, 2003, 75: 1067–1074
8 Slysz G W, Lewis D F, Schriemer D C. Detection and identification of sub-nanogram levels of protein in a nanoLC-trypsin-MS system. J Proteome Res, 2006, 5: 1959–1966
9 Calleri E, Temporini C, Perani E, *et al.* Trypsin-based monolithic bioreactor coupled on-line with LC/MS/MS system for protein digestion and variant identification in standard solutions and serum samples. J Proteome Res, 2005, 4: 481–490
10 Sakai-Kato K, Kato M, Toyooka T. On-line trypsin-encapsulated enzyme reactor by the sol-gel method integrated into capillary electrophoresis. Anal Chem, 2002, 74: 2943–2949
11 Slysz G W, Schriemer D C. Blending protein separation and peptide analysis through real-time proteolytic digestion. Anal Chem, 2005, 77: 1572–1579
12 Duan J C, Sun L L, Liang Z, *et al.* Rapid protein digestion and identification using monolithic enzymatic microreactor coupled with nano-liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr A, 2006, 1106: 165–174
13 Ma J F, Qiao X Q, Deng Q L, *et al.* Organic-inorganic hybrid silica monolith based immobilized trypsin reactor with high enzymatic activity. Anal Chem, 2008, 80: 2949–2956
14 Han M J, Lee S Y. The *Escherichia coli* proteome: Past, present, and future prospects. Microbiol Mol Biol R, 2006, 7: 362–439